PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 1792.002PC02	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US99/26238	International filing date *(day/month/year)* 08 NOVEMBER 1999	(Earliest) Priority Date *(day/month/year)* 06 NOVEMBER 1998
Applicant STERRENBELD BIOTECHNOLOGIE NORTH AMERICA, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the **title**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26238

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12P 21/02; C12N 15/19; C07K 14/505

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.4, 70.1, 71.1, 471, 320.1, 325, 358, 360, 365, 365.1, 252.3, ; 530/351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 5,618,698 A (LIN) 08 APRIL 1997 (08.04.97), see especially column 10, lines 16-56, column 21, lines 54-63, column 26, lines 38-67 and claims .	1, 3, 6-12, -------- 4-5, 13-14
X - Y	WO 86/03520 A1 (GENETICS INSTITUTE, INC.) 19 JUNE 1986 (19.06.86), especially see page 22, lines 10-20, page 23 line 35 through page 24 line 5 and claims.	1, 3, 6-12 -------- 4-5, 13-14
X - Y	Database Caplus on STN, Columbus (OH): Chemical Abstracts 1997:511352, HAN et al. Cloning of a Human Erthropoietin cDNA and its Expression in COS-7 cells. Shengwa Gongcheng Xuebao. 1996, Vol.12, No.4, pages 394-399, see abstract.	1, 3, 6-12 ---------- 4-5, 13-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 24 JANUARY 2000	Date of mailing of the international search report 07 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FOZIA HAMUD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26238

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,010,002 A (LEVINSON et al.) 23 April 1991 (23.04.91), see column 6, line 22 through column 7, line 56.	1, 3-14
A	US 4,703,008 A (LIN) 27 OCTOBER 1987 (27.10.87), see entire document.	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26238

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.4, 70.1, 71.1, 471, 320.1, 325, 358, 360, 365, 365.1, 252.3, ; 530/351

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

West, US patent full, STN via medline, embase, caplus, search terms: erythropoietin or epo and recombinant production, with viral promoter and viral terminator, expression vectors: pVEX1, pDHFR, cell culture, methotrexate, increased or improved yield.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
1909.002PC02		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/26238	08 NOVEMBER 1999	06 NOVEMBER 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant STERRENBELD BIOTECHNOLOGIE NORTH AMERICA, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☒ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30 MAY 2000	16 FEBRUARY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Dorothea Lawrence for FOZIA HAMUD
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/26238

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-27
pages NONE , as originally filed
pages NONE , filed with the demand
, filed with the letter of

[X] the claims:
pages 28-29
pages NONE , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
, filed with the letter of

[X] the drawings:
pages 1-4
pages NONE , as originally filed
pages NONE , filed with the demand
, filed with the letter of

[X] the sequence listing part of the description:
pages NONE
pages NONE , as originally filed
pages NONE , filed with the demand
, filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/26238

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	2, 4, 13, 14	YES
	Claims	1, 3, 5-12	NO
Inventive Step (IS)	Claims	2, 4, 13 , 14	YES
	Claims	1, 3, 5-12	NO
Industrial Applicability (IA)	Claims	1-14	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1, 3, 5-12 lack novelty under PCT Article 33(2) as being anticipated by LIN et al (5,618,698). Document U.S Patent 5,618,698 teaches a method of recombinantly producing erythropoietin (EPO) by transfecting a host cells(CHO, COS-1) with a vector comprising a nucleotide sequence encoding the EPO polypeptide of SEQ ID NO:1, claimed in the instant case, SV40 promoter and terminator, further comprising pDHFR vector. The expression system used in this document employed the selectable marker DHFR, (column 25, line 50 through column 26, line 37). To increase the quantity of EPO produced, host cells were cultured in the presence of methotrexate, the produced EPO polypeptide was then recovered,(see column 26, line 39 through column 27, line 16). Thus U.S Patent 5,618,698 anticipates instant claims 1, 3 and 5-12.

Claims 1, 3, 5, 7-12 lack novelty under PCT Article 33 (2) as being anticipated by WO 86/03520 and HAN et al, because these documents teach recombinant production of EPO using host cells transfected with a polynucleotide encoding EPO, SV40 promoter and terminator sequences, pDHFR as a selectable marker.

Claims 2,4 and 13-14 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the host cell of claim 2, or a host cell comprising a vector which comprises a nucleotide sequence obtained from human white blood cells, encoding the EPO of SEQ ID NO:1, a viral promoter and a viral terminator, wherein said vector also comprises pVex 1, or a host culture that produces 50 mg/L of EPO per day.

---------- NEW CITATIONS ----------

NONE

International application No.
PCT/US99/26238

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12P 21/02; C12N 15/19; C07K 14/505 and US Cl.: 435/69.4, 70.1, 71.1, 471, 320.1, 325, 358, 360, 365, 365.1, 252.3, ; 530/351

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12P 21/02, C12N 15/19, C07K 14/505 — A1

(11) International Publication Number: **WO 00/28066**

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/US99/26238

(22) International Filing Date: 8 November 1999 (08.11.99)

(30) Priority Data:

P98 01 05609	6 November 1998 (06.11.98)	AR
P99 01 00679	23 February 1999 (23.02.99)	AR

(71) Applicant *(for all designated States except US)*: STERREN-BELD BIOTECHNOLOGIE NORTH AMERICA, INC. [US/US]; 1209 Orange Street, Wilmington, DE 19801 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CARCAGNO, Carlos, Miguel [AR/AR]; Guevara 275, 1427 Buenos Aires (AR). CRISCUOLO, Marcelo [AR/AR]; Guevara 349, Capital Federal (AR). MELO, Carlos [AR/AR]; Alsina 2737, Del Viso, Pcia Buenos Aires (AR). VIDAL, Juan, Alejandro [AR/AR]; Guise 1760, 9th floor, Apartment A, 1425 Buenos Aires (AR).

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: HOST CELLS EXPRESSING RECOMBINANT HUMAN ERYTHROPOIETIN

(57) Abstract

The gene coding for human erythropoietin (EPO) was obtained from human genomic DNA. The gene used does not include sequences from regions at *i* 5' of the first translated ATG and *ii* 3' of the stop codon of the EPO gene. The gene was cloned into an expression plasmid for eukaryotic cells that have as sole expression control elements the early promoter of the SV40 virus and its polyadenylation signal. Recombinant cells resulting from transfection with genetic constructs used provide an unexpectedly high level of protein expression of 50 mg of recombinant EPO per liter of culture medium per day.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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Host Cells Expressing Recombinant Human Erythropoietin

Background of the Invention

Field of the Invention

The present invention relates, in general, to a host cell and a vector comprising a nucleotide sequence coding for recombinant human erythropoietin (EPO). In particular, the expression vector comprises only one promoter that regulates the EPO expression. The present invention also refers to a method of producing EPO and the EPO thus produced.

Background Information

EPO is a glycoprotein that stimulates erythroblast differentiation in the bone marrow, thus increasing the circulating blood erythrocyte count. The mean life of erythrocytes in humans is 120 days and therefore, a human being loses 1/120 erythrocytes each day. This loss must be continuously restored to maintain an adequate level of red blood cells.

The existence of EPO was first postulated by the turn of the century and was definitely proved by Reissman and Erslev early in the '50s. See Carnot, *et al., C.R. Acad. Sci. (France)143*:384-386 (1906); Carnot, *et al., C.R. Acad. Sci. (France), 143*:432-435 (1906); Carnot, *et al., C.R. Soc. Biol., 111*:344-346 (1906); Carnot, *C.R. Soc. Biol., 111*:463-465 (1906); Reissman, *Blood 5*:372-380 (1950) and Erslev, *Blood 8*:349-357 (1953). Reissman and Erslev's experiments were promptly confirmed by other researchers. See Hodgson, *et al., Blood, 9*:299-309 (1954); Gordon, *et al., Proc. Soc. Exp. Biol. Med., 86*:255-258 (1954) and Borsook, *et al., Blood, 9*:734-742 (1954).

The identification of the EPO production site in the organism was an issue of debate. Successive experiments led to the identification of the kidney as the main organ and peritubular interstitial cells as the synthesis site. See Jacobson, *et al., Nature, 179*:633-634 (1957); Kuratowska, *et al., Blood, 18*:527-534 (1961);

Fisher, *Acta Hematol., 26*:224-32 (1961); Fisher, *et al., Nature, 205*:611-612 (1965); Frenkel, *et al., Ann. N.Y. Acad. Sci., 149*:292-293 (1968); Busuttil, *et al., Proc. Soc. Exp. Biol. Med., 137*:327-330 (1971); Busuttil, *Acta Haematol., (Switzerland), 47*:238-242 (1972); Erslev, *Blood, 44*:77-85 (1974); Kazal, *Ann. Clin. Lab. Sci., 5*:98-109 (1975); Sherwood, *et al., Endocrinology, 99*:504-510 (1976); Fisher, *Ann. Rev. Pharmacol. Toxicol., 28*:101-122 (1988); Jelkmann, *et al., Exp. Hematol., 11*:581-588 (1983); Kurtz, *et al., Proc. Natl. Acad. Sci. (USA), 80*:4008-4011 (1983); Caro, *et al., J. Lab. Clin. Med., 103*:922-931 (1984); Caro, *et al., Exp. Hematol., 12*:357 (1984); Schuster, *et al., Blood, 70*:316-318 (1986); Bondurant, *et al., Mol. Cell. Biol., 6*:2731-2733 (1986); Schuster, *et al., Blood, 71*:524-527 (1988); Koury, *et al., Blood, 71*:524-527 (1988); Lacombe, *et al., J. Clin. Invest., 81*:620-623 (1988); Koury, *et al., Blood, 74*:645-651 (1989).

A smaller proportion, ranging from 10% to 15% of total EPO, is produced by the liver in adults. See Naughton, *et al., J. Surg. Oncol., 12*:227-242 (1979); Liu, *et al., J. Surg. Oncol., 15*:121-132 (1980); Dornfest, *et al., Ann. Clin. Lab. Sci., 11*:37-46 (1981); Dinkelaar, *et al., Exp. Hematol., 9*:796-803 (1981); Caro, *et al., Am. J. Physiol., 244*:5 (1983); Dornfest, *et al., J. Lab. Clin. Med., 102*:274-285 (1983); Naughton, *et al., Ann. Clin. Lab. Sci., 13*:432-438 (1983); Jacobs, *et al., Nature, 313*:806-810 (1985); Erslev, *et al., Med. Oncol. Tumor. Pharmacother., 3*:159-164 (1986). The EPO produced is directly proportional to the extent of tisular hypoxia and its expression rises by increasing the number of the EPO producing cells.

EPO has shown great efficiency in the treatment of anemia, especially anemia derived from renal failure. See Eschbach, *et al., N. England J. of Med., 316*:73-78 (1987); Krane, *Henry Ford Hosp. Med. J., 31*:177-181 (1983). Its therapeutical usefulness, however, has been limited due to the unavailability of a massive production method. The quantity and quality of the EPO obtained by the extractive systems known were insufficient. Recently, the use of recombinant DNA technology has made it possible to obtain large amounts of proteins. The application of these techniques to eukaryotic cells has allowed a large-scale production of EPO. See patents US 5,688,679 (to Powell), US 5,547,933 (to Lin),

US 5,756,349 (to Lin), US 4,703,008 (to Lin) and US 4,677,195 (to Hewick et al.).

At the present, recombinant DNA techniques are widely known and used. These techniques involve the use of genetic elements such as DNA fragments and enzymes to assemble and transfer genetic constructions for the production of recombinant proteins. The recombinant DNA techniques also facilitate the study of biological mechanisms. See Frank-Kamenetskii, "Unraveling DNA" [Samaia Glavnaia Molekula] (Addison Wesley Longman Inc., Reading, Massachusetts, 1997); Brown, "Gene Cloning" (Chapman & Hall, London, England, 1995); Watson, et al., "Recombinant DNA", 2nd Ed. (Scientific American Books, New York, New York, 1992); Alberts et al., "Molecular Biology of the Cell" (Garland Publishing Inc., New York, New York, 1990); Innis et al., Eds., "PCR Protocols. A Guide to Methods and Applications" (Academic Press Inc., San Diego, California, 1990); Ehrlich, Ed., "PCR Technology. Principles and Applications for DNA Amplification" (Stockton Press, New York, New York, 1989); Sambrook et al., "Molecular Cloning. A Laboratory Manual" (Cold Spring Harbor Laboratory Press, 1989); Bishop et al., "Nucleic Acid and Protein Sequence. A Practical Approach" (IRL Press 1987); Reznikoff, Ed., "Maximizing Gene Expression" (Butterworths Publishers, Stoneham, Massachusetts, 1987); Davis et al., "Basic Methods in Molecular Biology" (Elsevier Science Publishing Co., New York, New York, 1986); Watson, "The Double Helix" (Penguin Books USA Inc., New York, New York, 1969).

Summary of the Invention

The claimed invention comprises an eukaryotic cell line that produces recombinant human EPO, obtained by means of its transfection with an expression vector that comprises a gene coding for human EPO. The vector further comprises an unique promoter and terminator as expression control elements. SEQ ID NO:1 identifies the EPO amino acid sequence codified by the gene used.

The invention provides a host cell comprising a vector which comprises a nucleotide sequence encoding the erythropoietin polypeptide consisting of the amino acid sequence in SEQ ID NO:1, a viral promoter and a viral terminator.

The invention further provides a method for producing an EPO polypeptide, comprising culturing the above host cell under such conditions that said polypeptide is expressed and recovered.

One of the advantages of this invention is that the EPO coding gene utilized does not include non-coding fragments of the 5' and 3' regions. However, the system claimed produces an unexpectedly high amount of EPO.

An additional advantage of this invention is the use of expression vectors comprising only one promoter, which exhibit a high EPO productivity. By utilizing the claimed method, it is possible to obtain more than 50 mg of EPO per liter of cell culture per day, that is, over five times the EPO production level claimed by the best method reported so far utilizing one promoter.

The combination of the EPO coding gene claimed in this invention and a simple promoter showed, surprisingly, to operate efficiently, resulting in a stable EPO producing cell. The transfected cells yielded an amount of EPO comparable to, or even higher than, those reported using in theory more adequate, though more complex and difficult to manipulate, genetic constructions.

An additional advantage of the claimed invention is the cotransfection with two vectors that confer different resistance, thus simplifying and facilitating the selection, genetic amplification and maintenance of the cotransfected EPO producing cells.

Further objects and advantages of the present invention will be clear from the description that follows.

Brief Description of the Figures

Figure 1 illustrates polyacrylamide gel (SDS-PAGE) analysis of an EPO sample obtained following the method described after purification. In lanes 1, 4 and 7, molecular weight markers were loaded. In lanes 2, 3, 5 and 6, different amounts of pure EPO obtained according to the claimed procedure were run. The

purity of the product obtained and the apparent molecular weight exceeding 30 kDa is coincident with the one reported for urinary human EPO as could be clearly observed.

Figure 2 illustrates a Western blot analysis of an EPO sample obtained according to the method described. Identity of the EPO produced is assessed, since it is recognized by a monoclonal antibody against human EPO. In lanes 1 and 2, a human EPO standard and molecular weight markers were loaded, respectively. EPO samples obtained according to the claimed method were loaded in lanes 3 to 5.

Figure 3 shows a SDS-PAGE analysis of a pure EPO sample obtained according to the method described, and further treated with glycanases. Molecular weight markers were loaded in lanes 1, 4 and 8. Lanes 2 and 7 correspond to untreated EPO. In lane 3, O-glycanase treated EPO was loaded; the presence of an O-glycosilation site is verified. In lane 5, N-glycanase partially digested EPO was loaded. The presence of 3 N-glycosilated molecules with molecular weights as expected for EPO can be verified. Lane 6 was loaded with EPO digested with N-glycanase, and the expected molecular weight for the wholly deglycosilated protein was obtained.

Figure 4 illustrates a survey of the isoelectric points in pure EPO samples produced according to the method described. EPO samples were run in lanes 2, 3 and 4, isoelectric point markers in lanes 1 and 5. The presence of isoforms corresponding to EPO are verified, showing an isoelectric point range of 3.0 to 4.5.

Detailed Description of the Invention

The biological basis of recombinant DNA technology could be summarized as follows.

DNA (deoxyribonucleic acid) is the genetic material of all living cells and some viruses. Polymeric chains of four different nucleotides form the DNA, each of them being a purine or pyrimidine bound to a desoxyribose. The sugar moiety

is in turn linked to a phosphate group. These four nucleotides are: adenine (A), cytosine (C), guanine (G) and thymine (T).

The DNA chains are formed by phosphotriester linkages between nucleotides, where the phosphate in position 5' of the deoxyribose of one nucleotide is bound to the 3' position of the deoxyribose of the previous nucleotide. Synthesis *in vivo* occurs from 5' to 3', which is the conventional direction adopted to describe DNA sequences.

Functional DNA is presented as a double helix of complementary bases, where chains are held together by hydrogen bonds formed between A's and C's of one chain and T's and G's of the complementary chain, respectively. This is the reason why they are referred to as "base pairs".

The chains are also antiparallel, that is, the 5' end of each helix is matched to the 3' end of the other, as depicted below:

5' ---TACGTAC---3'
3' ---ATGCATC---5'

For protein synthesis to occur certain DNA coding regions are first transcribed to messenger RNA (mRNA). The mRNA is translated in turn into a protein. Each of the DNA coding regions is called a gene.

The synthesis of RNA (ribonucleic acid) chains involves the transcription of certain gene regions by enzymes called RNA polymerases. An antiparallel RNA chain, complementary to the DNA template, is thus obtained. Each A from DNA will correspond to a U in the RNA, each C to a G, each G to a C and each T to an A. The RNA molecule is also characterized because it is less stable than DNA. In addition, the sugar moiety in RNA is ribose instead of desoxiribose as in DNA. RNA is further distinguished from DNA by the substitution of uracyl (U) in place of thymine (T).

Matrix DNA	5'---------- ACGTAG ------3'
Synthesized mRNA	3'---------- UGCAUC ------5'

In eukaryotic cells, synthesized mRNA is processed in the nuclei (splicing) to result in mature mRNA. This process is not verified in bacteria.

Mature mRNA is then taken as matrix to be translated into a protein, in a process where transfer RNA (tRNA, small RNA chains that carry amino acids and align them specifically to form a protein) and ribosomes are the main participants. Three mRNA bases (triplet or codon) code each amino acid. For instance, the AUG sequence in mRNA codes for the amino acid methionine. The mRNA chains are thus translated into a specific peptide sequence, which finally folds into an active protein. The protein synthesis is called "expression."

The amount of protein expressed depends, among other factors, on the presence of certain DNA regions called promoters, which affect the rate at which the expression process occurs. In addition, there are DNA sequences that indicate the termination of transcription (terminators) and codons which indicate the end of translation (stop codons).

DNA technology involves the isolation of DNA fragments, either natural or synthetic, and their insertion into cells (i.e. bacteria, yeast, insect and mammalian cells) to render them capable of producing heterologous proteins such as EPO. The proteins obtained by recombinant DNA technology are called recombinant proteins.

The application field of recombinant DNA technology is not limited to cultured cells, since genes can also be incorporated into multicellular organisms (i.e. plants, insects, mammals and fish).

The expression of heterologous proteins requires the following elements:

- A gene coding for the desired protein. The gene may be obtained using different techniques, such as;
- Isolation from genomic DNA libraries.
- *In vitro* synthesis of DNA chains. There are commercially available equipments that synthesize relatively short DNA strands, making it possible to synthesize a gene *in vitro*.
- Amplification. Technology that allows to replicate several times a DNA fragment, such as a gene.

- Others, i.e. as obtained from cDNA libraries synthesized from mRNA.
- Active promoters to express a protein in the cell of interest.
- Proper terminators so that transcription is correctly terminated.
- Vectors. Genetic constructions such as plasmids or viruses that direct the gene with its promoter and terminator towards the inner region of the cell of interest incorporating the gene either in a chromosome or extrachromosomally. In certain cases, the incorporated gene may remain indefinitely in the cell and be transmitted to its progeny, or be lost in a relatively short term. There are multiple vector systems such as plasmids, and natural or modified viruses. It is also possible to use physical means of DNA introduction such as cell or nuclei microinjection. Viruses and plasmids are obtained from nature and are genetically modified *in vitro* to achieve the desired characteristics.
- Others. Additionally, other genetic elements may be necessary to improve the selection of cells receiving the gene (i.e. another gene conferring resistance to antibiotics) or to amplify the number of copies of the gene in each cell (genetic amplification).

Vectors and genes should be as simple as possible to reduce the time necessary to develop the system. A fundamental consideration is that genetic simplicity should not disregard the productivity or quality of the protein produced.

To achieve the expression of the protein of interest, the appropriate corresponding gene is transfected with the proper vectors within the host cell. Transfection may be done by different techniques such as electroporation, precipitation with calcium phosphate and the use of lyposomes, among other techniques available.

The gene of interest may be associated to other genes already known to confer resistance, for instance, to antibiotics such as geneticin, or to toxic agents such as methotrexate (MTX). This association allows the selection of the transfected cells in a stable manner, that is, those selected are capable of reproducing and transmitting the gene of interest to their progeny. Association

also permits to select the recombinant cells showing the highest expression level of the protein of interest.

The recombinant product thus obtained is identified by its molecular weight, amino acid sequence and biological activity, among other applicable assays.

The tools (i.e. restriction enzymes) and techniques that gave rise to recombinant DNA technology were first developed in the early '70s and were followed by an intense and widespread utilization. More particularly, the genetic engineering techniques utilized presently to produce EPO involve the following:

1. The use of EPO genes including fragments of non-coding regions located 5' of the first translated ATG and 3' of the stop codon of the gene. It is conventionally believed that the presence of expression control elements located in the non-coding regions of the gene is necessary to achieve a high production of EPO. See patent US 5,688,679 (to Powell).
2. The employment of expression vectors with different promoters, was based upon the premise that a combination of promoters induces a higher EPO production. Until now, the use of only one promoter included in the vector has resulted in a low level of protein expression. See patents US 4,703,008 (to Lin), US 4,677,195 (to Hewick et al.) and US 5,688,679 (to Powell). Average production of EPO using only one promoter is 200 μ/l/day. Maximum production of EPO reported using only one promoter is 10 mg/l/day.
3. The potential instability of the genetic systems due to the complexity of the genetic constructs utilized.

In order to obtain the claimed EPO producing cells, genomic DNA is first extracted from human white blood cells. The EPO coding gene is obtained from the isolated genomic DNA. To achieve this, the gene is amplified using adequate primers to prevent the occurrence of 5' and 3' non-coding regions of the EPO gene. These primers include restriction sites in their 5' ends that remain at both ends of the isolated gene to facilitate further cloning.

The amplified gene is next cloned in a bacterial vector and sequenced. Once the sequence obtained is verified, the gene is cloned into the Xho I-Hind III sites of an expression vector for eukaryotic cells harboring only the SV40 early promoter and its terminator. The vector confers resistance to geneticin and ampicillin.

The CHO cells are subsequently cotransfected with two vectors: 1) the EPO expression vector and 2) a vector that confers resistance to methotrexate. Stably transfected EPO producing cells are selected according to their resistance to geneticin. The level of EPO expression is monitored by the selection of amplified cells resistant to increasing concentrations of methotrexate.

Finally, clones are selected according to their productivity level as measured by radioimmunoassay. Culture supernatants of the most productive clones are used to test the identity of the EPO produced by SDS-PAGE, Western blot, glycanase treatment followed by SDS-PAGE, isoelectric focusing and a complete protein sequence analyses. The biological *in vivo* activity of the produced EPO is determined by an ex-hypoxic polycythemic mice assay using as reference the international standard for EPO standard.

Vectors and Host Cells

The present invention relates to vectors which include a nucleotide sequence encoding EPO, host cells genetically engineered with the recombinant vectors, and the production of EPO polypeptides or portions thereof by recombinant techniques.

Recombinant constructs may be introduced into host cells using well-known techniques such as infection, transduction, transfection, transvection, conjugation, electroporation and transformation. The vector may be, for example, a phage, plasmid, viral or retroviral vector.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a

charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

Preferred are vectors comprising *cis*-acting control regions to the polynucleotide of interest. Appropriate trans-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression, which may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda P_L promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating codon (AUG or GUG) at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. More preferably, two expression vectors will include a total of two markers. Such markers include methotrexate, dihydrofolate reductase or neomycin resistance. Preferred vectors confer resistance to methotrexate and neomycin-derived antiobiotics such as genetycin.

Especially preferred host cells are mammalian cells comprising CHO, COS, BHK, Namalwa and HeLa. Preferred host cells are CHO cells. Appropriate culture media and conditions for the above-described host cells are known in the art.

A preferred method of obtaining EPO from the host cells of the invention is culturing in media comprising insulin. Specifically, such culturing comprises separating the supernatant which comprises EPO and insulin from the host cells of the invention, concentrating the supernatant and freezing the concentrated product. Preferably, the culture media comprises between 0.5 mg and 20 mg insulin per liter of culture media.

Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Especially preferred vectors are pDHFR, and pVex 1. The pDHFR vector includes DNA encoding for the dehydrofolate reductase (DHFR) of mouse, whose expression is controlled by the early promoter of the SV40 virus and its polyadenylation signal. The pVex 1-EPO vector comprises the DNA encoding the EPO polypeptide in SEQ ID NO:1, an element conferring resistance to neomycin-derived antibiotics, an early promoter of the SV40 virus and the polyadenylation signal of the SV40 virus.

Suitable eukaryotic promoters for use in the present invention include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter. An especially preferred promoter is the viral SV40 early promoter.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other

methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, "Basic Methods in Molecular Biology," (1986).

An especially preferred method to effect introduction of the construct of the invention into a host cell is by calcium phosphate transfection.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually of a size ranging from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, either during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification.

The EPO protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography.

A preferred method of purifying the EPO produced by the cells of the invention comprises treating cell culture supernatants comprising EPO by a combination of the following steps: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography and (f) molecular exclusion chromatography. Preferably, said steps are performed in the following order: (a), (b), (c), (d), (e), and (f).

A preferred method of using the EPO produced by the cells of the present invention comprises lyophilization into a form suitable for injection into humans for treatment of disease. Specifically, the preferred lyophilization procedure comprises placing the EPO into a pharmaceutical composition, loading the first EPO composition into a container, wherein said container is at a temperature equal to or less than -30°C; incubating said EPO composition at a temperature equal to or less than -30°C under atmospheric pressure for a time equal to or greater than 4 hours; incubating said composition at a pressure of equal to or less than 30 absolute microns for a time equal to or greater than one hour; and raising the temperature equal to or less than 3°C per hour until reaching at least 25°C, while keeping pressure values equal to or less than 5 absolute microns.

A preferred pharmaceutical composition for lyophilization comprises EPO, sugar, salts and human albumin. An especially preferred composition for lyophilization comprises EPO, mannitol, NaCl, NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

Nucleic Acid Molecules

The host cells of the present invention may comprise vectors which comprise the EPO nucleic acid molecule from Lin, "DNA Sequences Encoding Erythropoietins," U.S. Patent No. 4,703,008, which is herein incorporated by reference, and variants thereof. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*,

Lewin, ed. Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the EPO protein or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding the EPO protein having the amino acid sequence shown in SEQ ID NO:1.

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the EPO polypeptide having the complete amino acid sequence in SEQ ID NO:1 or (b) a nucleotide sequence complementary the nucleotide sequence in (a).

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 97%, 98%, or 99% identical to a nucleic acid sequence encoding the EPO polypeptide will encode a polypeptide "having EPO protein activity." In fact, since all of each and every degenerate variant of these nucleotide sequences encode the same polypeptide, this will be clear to the skilled artisan. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having EPO activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that preserve functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J.U., *et al., supra,* and the references cited therein.

The invention claimed is better explained by the examples depicted below:

Examples

Example 1 Preparation of Human Genomic DNA

10 ml of blood were extracted from a clinically healthy human adult male subject and added to a test tube containing 10 mM EDTA (pH 8). The blood was transferred in 5 ml aliquots to two 50 ml test tubes, to which 45 ml of a solution containing 0.3 M of saccharose, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 1% Triton X 100 was added.

The solutions were then incubated on ice for 10 minutes and centrifuged for 10 minutes at 1,000 g at 4°C. The supernatants were discarded and the pellets rinsed several times with a 0.075 M NaCl solution containing 0.025 M EDTA (pH 8), followed by centrifugation for 10 minutes at 1,000 g at 4°C.

The resulting pellets thus obtained were resuspended in 3 ml of a 10 mM Tris-HCl (pH 8), 400 mM NaCl, 2 mM EDTA (pH 8) solution. 200 μl of 10 % SDS (sodium dodecyl sulfate) and 500 μl K proteinase (1 mg/ml in 1 % SDS and 2 mM EDTA pH 8) were then added, and the solutions were incubated overnight at 37°C. After the addition of 1 ml of NaCl saturated solution to each test tube the solutions containing the genomic DNA were centrifuged at 2,500 g for 15 minutes.

Each supernatant was transferred to a 15 ml test tube where one volume of isopropanol was added. The test tubes were gently mixed by inversion and stored at room temperature until a DNA precipitate was formed. The genomic DNA was then recovered with a hook-end Pasteur glass pipette.

The DNA was placed in a 2 ml test tube, and 1 ml of 70 % ethanol was added. After one minute, the supernatant was discarded and the precipitate was let dry. After drying, the precipitate was dissolved in 500 μl of TE buffer (10 mM Tris-HCl pH 8 - 1 mM EDTA).

The concentration of the DNA solution was calculated by measuring the absorbance of a 1:1000 dilution of the solution at 260 nm. It was assumed that 50 μg of genomic DNA was equivalent to 1 OD unit. A solution containing 500 ng of genomic DNA per μl of TE buffer was prepared.

Example 2 ***Preparation of the EPO Construct***

The EPO construct was prepared from 500 ng of human genomic DNA obtained in Example 1. The following was added to 1 μl of the solution resulting from Example 1 placed on a 0.5 ml test tube: 400 ng of each of the EPO 1 and EPO 2 primers, an aqueous solution of 2.5 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) and 2.5 units of Taq DNA polymerase (Perkin Elmer) in a final volume of 100 l using the buffer recommended by the manufacturer. A thermal cycler was used and programmed for 30 cycles of: 1 minute at 93°C, 1 minute at 55°C and 3 minutes at 72°C. From this reaction, a DNA fragment of approximately 2,170 base pairs containing the EPO gene was obtained.

The nucleotide sequences of the primers utilized were as follows:

EPO 1: 5' GAATTCTCGAGATGGGGGTGCACGGTGAG 3' (SEQ ID NO:2). This primer corresponds to the first bases which were translated from the EPO gene with a site for the recognition of the Xho I enzyme and another site for the recognition of the Eco RI enzyme in the 5' end. These sites were used in the subsequent cloning steps.

EPO 2: 5' AAGCTTTCATCTGTCCCCTGTCCTGCA 3' (SEQ ID NO:3). This primer is complementary to the last translated bases and the stop codon of the EPO gene. A site for the recognition of the Hind III enzyme was added to the 3' end of the primer. This site was used in subsequent cloning steps.

The nucleotide sequence obtained was as follows (SEQ ID NO:4):

gaattctcgagatgggggtgcacggtgagtactcgcgggctgggcgctcccgccgcccgggtccctgtttgagcggggatttagcgcc
ccggctattggccaggaggtggctgggttcaaggaccggcgacttgtcaaggaccccggaagggggagggggtggggcagcctc
cacgtgccagcggggacttgggggagtccttggggatggcaaaaacctgacctgtgaaggggacacagtttgggggttgaggggaag
aaggtttgggggttctgctgtgccagtggagaggaagctgataagctgataacctgggcgctggagccaccacttatctgccagagggg
aagcctctgtcacaccaggattgaagtttggccggagaagtggatgctggtagctgggggtggggtgtgcacacggcagcaggattga
atgaaggccagggaggcagcacctgagtgcttgcatggttggggacaggaaggacgagctggggcagagacgtggggatgaaggga
agctgtccttccacagccacccttctccctccccgcctgactctcagcctggctatctgttctagaatgtcctgcctggctgtggcttctcctg
tccctgctgtcgctccctctgggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctggagaggtacctcttg
gaggccaaggaggccgagaatatcacggtgagacccccttccccagcacattccacagaactcacgctcagggcttcagggaactcctc
ccagatccaggaacctggcacttggtttggggtggagttgggaagctagacactgccccccctacataagaataagtctggtggccccaa
accatacctggaaactaggcaaggagcaaagccagcagatcctacggcctgtgggccagggccagagccttcagggacccttgactc
cccgggctgtgtgcatttcagacgggctgtgctgaacactgcagcttgaatgagaatatcactgtcccagacaccaaagttaatttctatgc
ctggaagaggatggaggtgagttccttttttttttttttccttttcttttggagaatctcatttgcgagcctgattttggatgaaagggagaatgat
cgggggaaaggtaaaatggagcagcagagatgaggctgcctgggcgcagaggctcacgtctataatcccaggctgagatggccgag
atgggagaattgcttgagccctggagtttcagaccaacctaggcagcatagtgagatcccccatctctacaaacatttaaaaaaattagtc
aggtgaagtggtgcatggtggtagtcccagatatttggaaggctgaggcgggaggatcgcttgagcccaggaatttgaggctgcagtga
gctgtgatcacaccactgcactccagcctcagtgacagagtgaggccctgtctcaaaaaagaaaagaaaaaagaaaaataatgagggc
tgtatggaatacattcattattcattcactcactcactcattcattcattcattcaacaagtcttattgcataccttctgtttgctcagctt
ggtgcttggggctgctgaggggcaggagggagagggtgacatgggtcagctgactcccagagtccactccctgtaggtcgggcagca
ggccgtagaagtctggcagggcctggccctgctgtcggaagctgtcctgcggggccaggccctgttggtcaactcttcccagccgtgg
gagcccctgcagctgcatgtggataaagccgtcagtggccttcgcagcctcaccactctcttcgggctctgggagcccaggtgagtagg
agcggacacttctgcttgcccttctgtaagaaggggagaagggtcttgctaaggagtacaggaactgtccgtattccttccctttctgtgg
cactgcagcgacctcctgttttctccttggcagaaggaagccatctcccctccagatgcggcctcagctgctccactccgaacaatcactg
ctgacactttccgcaaactcttccgagtctactccaatttcctccggggaaagctgaagctgtacacaggggaggcctgcaggacaggg
gacagatga***aagctt***

The first translated atg codon, as well as the tga "stop" codon, are underlined. The sequences of restriction sites utilized in the cloning are shown in bold italics. It should be noted that more than one codon may code for the same amino acid, and that consequently, the EPO protein could be translated from different mRNA templates having different nucleotide sequences but coding nevertheless for EPO.

Example 3 Cloning and Sequencing of the EPO Gene

A fragment of approximately 2,170 base pairs containing DNA coding EPO was purified. The ends of the DNA were blunted by treatment with the DNA polymerase Klenow's fragment and cloned in the Sma I site of a M13mp18 vector, following standard molecular biology techniques. The recombinant plasmids obtained were cut with Xho I and Hind III enzymes. The presence of the insert was verified by electrophoresis of the restriction fragments in a 0.8 % agarose gel stained with ethydium bromide. A positive clone (two bands, one having approximately 2,200 base pairs and the other one corresponding to the linearized vector) were selected. The inserted EPO gene was sequenced according to Sanger's technique using a T7 sequencing kit (Pharmacia). Some regions of the EPO gene were further sequenced with an automatic 370 A Applied Biosystems International sequencer. For each sequencing system the protocols recommended by the manufacturers were followed.

Example 4 Vectors for Eukaryotic Cells

1. Construction of pVex 1 Vector

The pVex1 vector was built following the standard molecular biology techniques. It consisted of:

a. Fragments of the bacterial pBR322 vector, which have a bacterial replication origin and confer resistance to ampicillin, for amplification and selection of the vector in *E. coli*.

b. Immediately downstream from a) follows an early promoter of the SV40 virus, which allowed the expression of the genes cloned at 3' from this element.

c. Immediately downstream from b) follow the Xho I and Hind III cloning sites, which allowed the insertion of the genes to be expressed.

d. Immediately downstream from c) follows the polyadenylation signal of the SV40 virus, which allowed the proper polyadenylation of the specific transcripts of the gene cloned in c).

e. Immediately downstream from d) follow the TK promoter and the gene coding for neomycin phosphotransferase with its polyadenylation signal. These elements allowed the selection of stably transfected cells through the use of neomycin and neomycin-derived antibiotics such as geneticin. The 3' end of e) is linked to the 5' end of a).

The pVex 1 vector was deposited on April 16, 1999 at DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, and was given accession number DSM 12776.

2. pDHFR Vector

The pDHFR vector confers resistance to ampicillin for selection in bacteria. This vector includes the cDNA coding for mice dihydrofolate reductase (DHFR), whose expression level is controlled by the SV40 virus early promoter and its polyadenylation signal. The EPO expression level achieved with the pVex 1-EPO (See Example 5) vector is enhanced several times by the amplification of the DHFR and EPO genes in a culture medium containing increasing concentrations of methotrexate (MTX).

The pDHFR vector was deposited on April 16, 1999 at DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, and was given accession number DSM 12777.

Example 5 Cloning of the EPO Coding Gene into an Expression Vector

The M13mp18 clone containing the EPO coding gene cloned as described in Example 3 was cut with Xho I-Hind III enzymes. The fragment thus obtained, of approximately 2.2 Kb, was isolated and cloned again using the same restriction sites of the pVex I vector. A positive pVex-EPO clone was isolated and analyzed showing that the EPO coding sequence SEQ ID NO: 4 did not change during the cloning procedures.

The previous examples were performed according to conventional molecular biology techniques. See Brown, "Gene Cloning", (Chapman & Hall, London, England, 1995); Watson, et al., "Recombinant DNA, 2nd Ed.", (Scientific American Books, New York, New York, 1992); Sambrook et al, "Molecular Cloning. A Laboratory Manual", (Cold Spring Harbor Laboratory Press, 1989); Bishop et al., "Nucleic Acid and Protein Sequence. A Practical Approach", (IRL Press, 1987); Davis et al., "Basic Methods in Molecular Biology", (Elsevier Science Publishing Co., New York, New York, 1986).

Example 6 Co-transfection and Amplification

A mutagenized CHO (Chinese Hamster Ovary) cell line, deficient in the DHFR-enzyme gene (CHO-DHFR$^-$), was used to facilitate gene amplification with MTX. During the entire process cells were grown in a 5% CO_2 atmosphere at 37°C.

The CHO cells were cotransfected following the calcium phosphate technique which, using a 90 mm diameter Petri dish, was as follows:

(1) The culture medium (alpha-MEM, with 10 % of fetal calf serum) was replaced with fresh medium 4-8 hours before transfection.

(2) 500 μL of 10 g/l HEPES (pH 7.1) solution, 500 μL of 16 g/l NaCl, 10 μl of a 35 mM Na_2HPO_4 and 10 μl of 35 mM of NaH_2PO_4 solution were added to a 5 ml test tube.

(3) In a separate 1.5 ml test tube, a solution with 60 μl of 2 M $CaCl_2$ and 10 μg of each DNA vector to be transfected (pVex-EPO and pDHFR) were

added. Water was added until a final volume of 500 µl was reached. The pDHFR plasmid described in Example 2 is based on the pBR 322 plasmid, which is ampicillin-resistant. The pDHFR plasmid can be replicated in *E. Coli* and has the DHFR gene cloned between the SV40 early promoter and its terminator. The pDHFR plasmid codes for the expression of the DHFR protein in CHO cells. This protein confers resistance to methotrexate, which can then be used to select cells showing high erythropoietin productivity.

(4) The solution containing DNA and $CaCl_2$ was added drop by drop to the test tube containing HEPES, while air was bubbled to obtain a rapid mixing and minimize local concentration. This method facilitated the formation of very small particles containing DNA and calcium phosphate which are more effectively incorporated by the cells.

(5) The solution was allowed to settle for 30 minutes and was added then to the Petri dish containing the cells.

(6) The solution was distributed among the cells by gentle shaking. The cells were left overnight in an incubator under a 5% CO_2 atmosphere at 37°C.

(7) Cells were rinsed twice with a PBS buffer (8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4, 0.24 g NaH_2PO_4, water was added to 1 liter and pH was adjusted to 7.4 with HCl). Fresh culture medium was then added.

The selection with geneticin (G 418) at a concentration of 600 g/ml begun 24 hours after transfection. The cells which incorporated the pVex-EPO plasmid were able to resist the antibiotic, while all others died after 25 days. Resistant colonies were selected and their productivity was assayed. The three most productive clones were selected from the isolated clones.

Taking advantage of the genetic constructions used in the invention, a selection was performed for each of the three clones using MTX as secondary selective agent at a 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M concentration. For that purpose, the culture medium was changed to alpha-MEM without nucleotides, supplemented with 10% dialyzed fetal calf serum. It is essential to perform the dialysis process according to the following protocol: 100 ml of serum were placed

in a dialysis bag with a 3,000 Da cut off (with a higher cut off, growth factors could be lost, and the cells would not be able to grow and reproduce), the bag was hermetically closed, completely immersed in a container with 5 liters of bidistilled water and left without agitation for 12 hours at 4°C. After this step, the water was discarded and changed, leaving the bag to stand for an additional 12 hour period at 4°C. The dialysis bag was then removed and the serum recovered. The dialysis during shorter periods, with smaller volumes or without water replacement, would be ineffective since any trace of nucleotides in the serum will affect the MTX selection adversely. In the other hand, dialysis for longer periods would also be ineffective because some proteins necessary for cell growth may precipitate preventing cell maturation.

Example 7 Isolation of High Productivity Clones

Clones that grew in 10^{-7} and 10^{-6} M of MTX were isolated and amplified in fresh alpha-MEM without nucleotides supplemented with 10% of dialyzed fetal calf serum. Once grown, the culture supernatant was assayed for the production and secretion of EPO. For that purpose, a specific immunoassay was used.

The process described above concluded with the selection of a clone of recombinant host cells producing 50,000 μg of erythropoietin/liter of culture medium per day.

The recombinant host cell described in this example was deposited on April 16, 1999 at DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany and given accession number DSM ACC2397.

The effectiveness of transcription process in the cell was verified as described in Example 8. The sequence of the obtained protein was identified following the procedure described in Example 9.

Example 8 ***Verification of the Specific Messenger RNA Sequence Produced by the Recombinant Cells***

1. Preparation of RNA from Cells

Total RNA was prepared from EPO producing cells, according to the following protocol:

A 90 mm diameter Petri dish having confluent cells was washed twice with 10 ml of PBS buffer. 2 ml of GTC buffer were then added and distributed evenly over the dish. The GTC buffer was composed of: 50 g guanidinum thiocyanate, 0.5 g N-Lauroylsarcosine, 2.5 ml 1 M sodium citrate (pH 7), 0.7 ml β-mercapthoethanol, 0.33 ml of 30% antifoam agent (SIGMA) and 100 ml H_2O q.s. (pH 7.0).

Cells were then lysed. The lysate resulted in a highly viscous solution. The solution was transferred to a 15 ml test tube, and the process above described was repeated once more using 2 ml of GTC buffer.

The 15 ml test tube was vigorously stirred for 1 minute to break the DNA. A cesium chloride gradient was then performed. For that purpose, 4 ml of a solution containing CsCl (95.97 g CsCl and 2.5 ml of 1 M Sodium acetate, (pH 5.4), and water was added until a volume of 100 ml was reached) were added to an ultracentrifuge test tube. Over this solution and without mixing, the suspension of the cells in GTC was then added. The test tube was next filled with GTC buffer and ultracentrifuged at 31,000 rpm for 20 hours at 20°C.

During centrifugation, the RNA was deposited at the bottom of the test tube forming a pellet and the DNA obtained showed a band in the middle of the cesium chloride gradient. The supernatant was discarded to eliminate thoroughly the DNA. The RNA-containing pellet was let dry for 5 minutes and was dissolved then in 200 μl of water and transferred to a 1.5 ml test tube. 200 μl of 0.4 M Sodium acetate, (pH 4.8) and 2 volumes of ethanol were then added, the resulting solution was thoroughly mixed and left to settle for 30 minutes at –80°C. The solution was then centrifuged in a microcentrifuge at 14,000 rpm for 15 minutes, the supernatant was discarded and the precipitate was rinsed with 1 ml 80 %

ethanol. The pellet was dried and redissolved in 100 μl of water. The concentration of a 1:100 dilution of the RNA solution was measured at 260 nm (1 OD unit corresponds approximately to 40 μg of RNA). All the solutions and elements used were RNase-free.

2. Preparation of cDNA

Specific cDNA was prepared following the directions of a kit intended for that purpose (cDNA Synthesis System Plus, Amersham - cat. RPN 1256). The EPO 2 oligonucleotide was the primer used.

3. Cloning of cDNA Coding for EPO

Five percent of the cDNA thus obtained was amplified using 400 ng of each the EPO 2 and EPO 3 oligonucleotides, 2.5 mM of each deoxynucleotide in the proper buffer, and 2.5 units of Taq DNA polymerase, in a total volume of 100 μl. 35 amplification cycles were performed as follows: 1 minute at 93°C, 1 minute at 55°C and 1 minute at 72°C.

EPO 3 was synthesized as described for EPO 1 and EPO 2, and its sequence (5' GAATTCCATGGGGGTGCACGAATGTCC 3') (SEQ ID NO:5) corresponded to the first 20 bases coding for the EPO cDNA, and one site for the recognition of the Eco RI enzyme. The Eco RI enzyme recognition site was added to facilitate subsequent cloning steps.

A fragment of approximately 600 base pairs was obtained and cloned in M13mp18 and M13mp19 vectors. Using the Sanger's sequencing method, the insert was sequenced in both directions to obtain the complete sequence.

Due to the high autocomplementarity of some regions of the gene, which gives rise to many and very ambiguous compressions in the autoradiography, a sequencing kit using Taq DNA polymerase and modified bases was used. Lower quality results were obtained, but the compressions were resolved. The kit used was the Pharmacia-LKB Biotechnology *Gene aTaq*.

The complete sequence of the human erythropoietin cDNA was isolated and cloned, showing to code for EPO. Consequently, the gene cloned in the cells and its transcription product were found complete and its sequence correct for EPO.

Example 9 Analysis of the EPO Produced

The EPO obtained by culturing the host cells as illustrated in the preceding example was further purified to undergo various quality and identificatory assays.

In a denaturing SDS-PAGE gel the EPO was identified as a wide band of molecular weight over 30 kDa as expected for EPO. See Figure 1. The band was recognized by monoclonal and polyclonal antibodies against human EPO in a "Western blot" assay as expected for EPO. See Figure 2. The treatment with glycanases proved the existence of the glycosidic chains in the extent and size as expected for EPO. See Figure 3. The EPO produced was shown to be composed of a series of species ranging isoelectric points from 3.0 to 4.5 as expected for EPO. See Figure 4.

The complete amino acid sequence of the isolated protein, purified from the culture supernatant of transfected cell lines showed total homology with natural human erythropoietin whose 165 amino acid sequence is as follows (SEQ ID NO:1):

NH_2---	Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp
	Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu
	Leu	Glu	Ala	Lys	Glu	Ala	Glu	***Asn***
	Ile	Thr	Thr	Gly	Cys	Ala	Glu	His
	Cys	Ser	Leu	Asn	Glu	***Asn***	Ile	Thr
	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe
	Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val
	Gly	Gln	Gln	Ala	Val	Glu	Val	Trp
	Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu

Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu
Leu	Val	***Asn***	Ser	Ser	Gln	Pro	Trp
Glu	Pro	Leu	Gln	Leu	His	Val	Asp
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser
Leu	Thr	Thr	Leu	Leu	Arg	Ala	Leu
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser
Pro	Pro	Asp	Ala	Ala	***Ser***	Ala	Ala
Pro	Leu	Arg	Thr	Ile	Thr	Ala	Asp
Thr	Phe	Arg	Lys	Leu	Phe	Arg	Val
Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys
Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala
Cys	Arg	Thr	Gly	Asp----COOH			

The presence of the four glycosilation sites along the 165 amino acid chain, as well as the complex carbohydrate structure, and in particular, the sialic acid terminal residues, which characterize EPO were verified. These results were further supported by a biological activity assay of the produced protein by an ex-hypoxic polycythemic mice test which showed complete concordance with the international EPO standard.

The productivity achieved, measured by a specific immunoassay, was 50 mg EPO per liter of culture medium per day.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A host cell comprising a vector which comprises:

(a) a nucleotide sequence encoding the erythropoietin polypeptide consisting of the amino acid sequence in SEQ ID NO:1;

(b) a viral promoter; and

(c) a viral terminator.

2. The host cell deposited as DSM ACC2397.

3. The host cell of claim 1, wherein said viral promoter and viral terminator comprises an early promoter and terminator of a SV40 virus.

4. The host cell of claim 1, wherein said vector comprises pVex 1.

5. The host cell of claim 1, further comprising a pDHFR vector.

6. The host cell of claim 4, wherein said host cell is resistant to neomycin-derived antibiotics and methotrexate.

7. The host cell of claim 1, wherein said host cell is a mammalian cell.

8. The host cell of claim 7, wherein said mammalian cell is selected from the group comprising: CHO, COS, BHK, Namalwa, HeLa, Hep3B, Hep-G2 or other mammalian cells.

9. The host cell of claim 1, wherein said host cell comprises a CHO or COS cell.

10. The host cell of claim 1, wherein said host cell comprises a CHO cell.

11. A method for producing an EPO polypeptide, comprising culturing the host cell of claim 1 under conditions such that said polypeptide is expressed and recovered.

12. The method of claim 11, wherein such conditions comprise exposure to methotrexate.

13. The method of claim 9, wherein said culture produces more than 50 mg of EPO per liter of culture medium per day.

14. The host cell of claim 1, wherein said nucleotide sequence is obtained from human white blood cells.

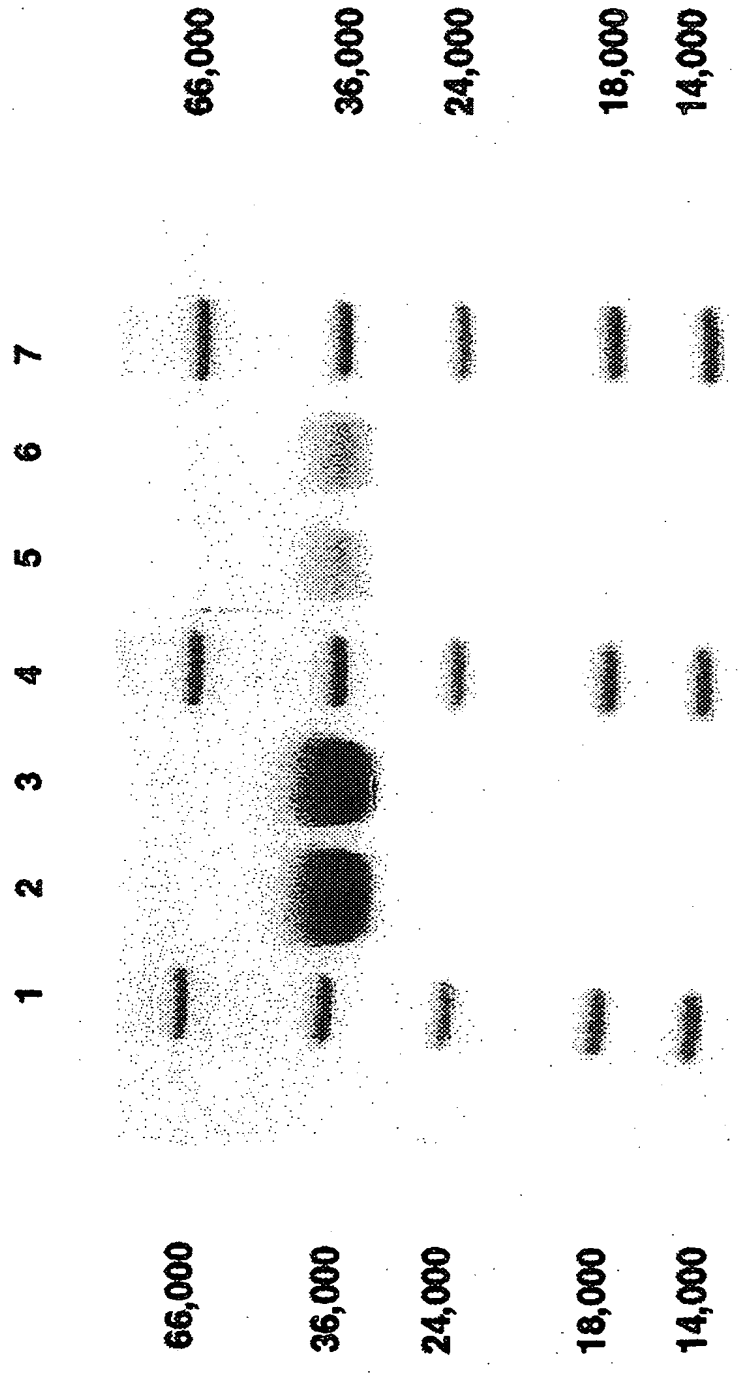
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FIG. 1

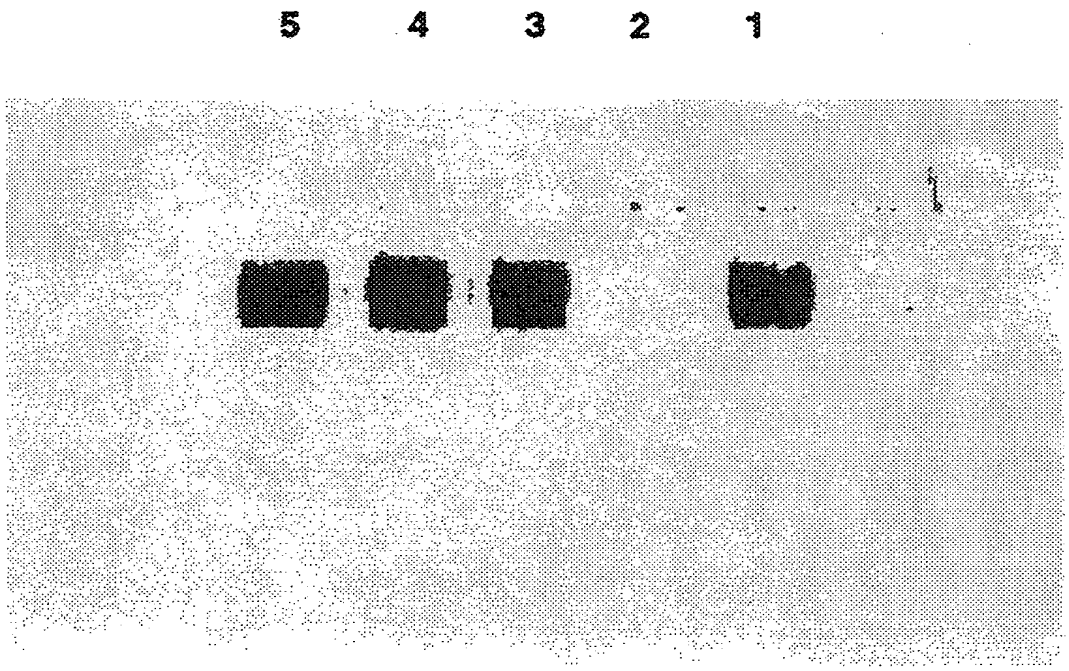

FIG.2

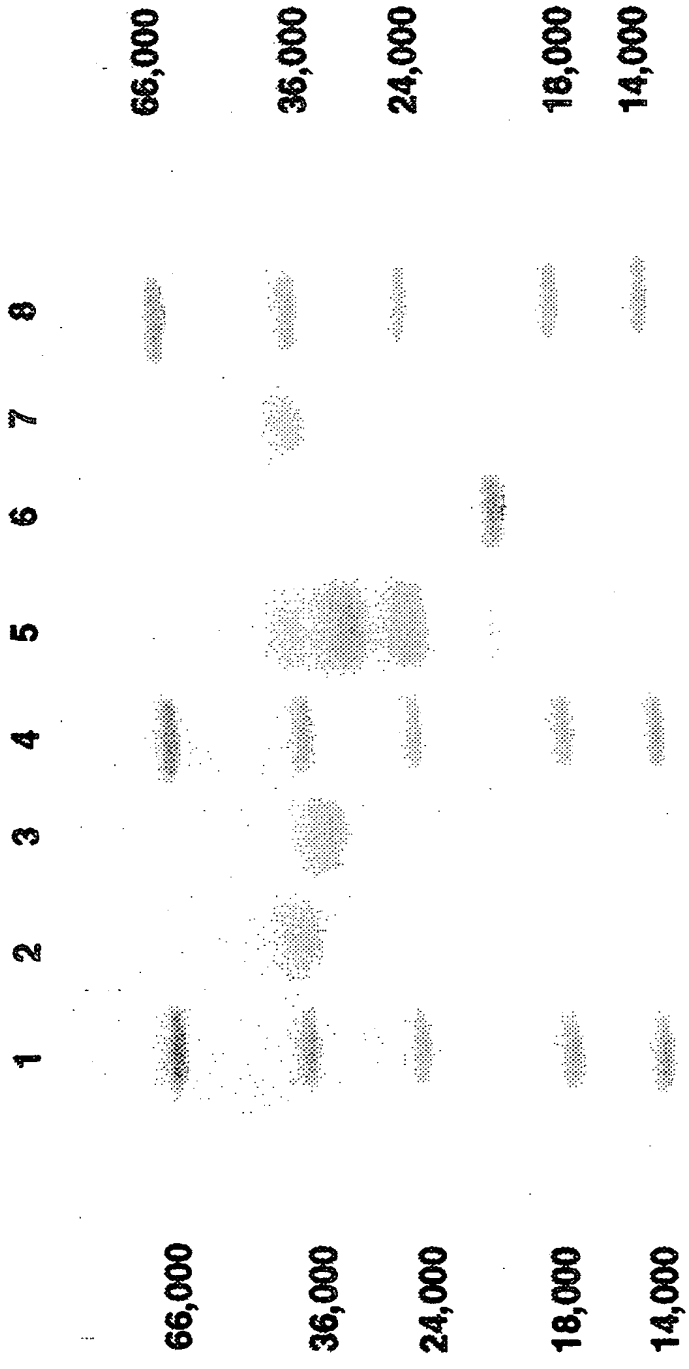
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FIG.3

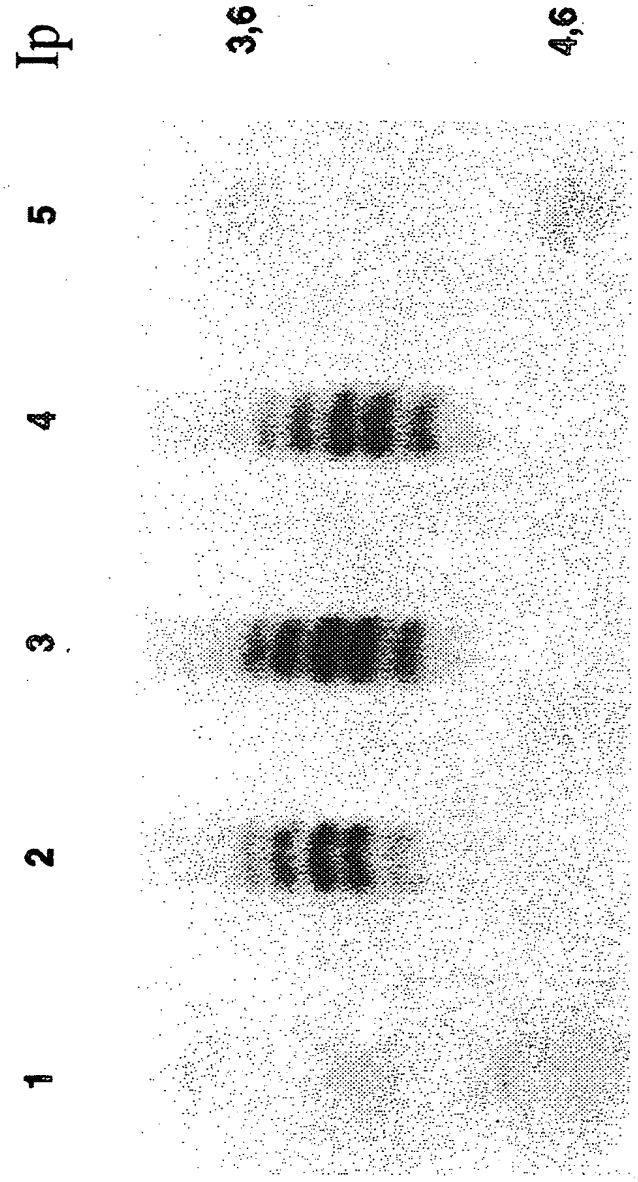

FIG.4

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Carcagno, Carlos Miguel
Criscuolo, Marcelo
Melo, Carlos
Vidal, Juan Alejandro

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Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 23, line 23.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit	Accession Number
16 April 1999 (16.04.99)	DSM ACC2397

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

MC2 20 22/12/98

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

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Authorized officer	Authorized officer

Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 20. line 27.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit	Accession Number
16 April 1999 (16.04.99)	DSM 12777

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

pDHFR

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 20, line 14.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit	Accession Number
16 April 1999 (16.04.99)	DSM 12776

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

pVex 1

In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

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☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Deposit: MC2 20 22/12/98
Accession Number: DSM ACC2397
Page: 1

AUSTRALIA

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

CANADA

The applicant hereby requests that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

DENMARK

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the National Board of Patents and Registration or any person approved by the applicant in the individual case.

ICELAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the Icelandic Patent Office), or has been finally decided upon by the Icelandic Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

Deposit: MC2 20 22/12/98
Accession Number: DSM ACC2397
Page: 2

NETHERLANDS

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

NORWAY

The applicant hereby requests that, until the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Norwegian Patent office or any person approved by the applicant in the individual case.

SINGAPORE

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

SWEDEN

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

UNITED KINGDOM

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AUSTRALIA

The applicant hereby gives notice that the furnishing of a sample of a microorganism shall only be effected prior to the grant of a patent, or prior to the lapsing, refusal or withdrawal of the application, to a person who is a skilled addressee without an interest in the invention (Regulation 3.25(3) of the Australian Patents Regulations).

CANADA

The applicant hereby requests that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

DENMARK

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the National Board of Patents and Registration or any person approved by the applicant in the individual case.

ICELAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the Icelandic Patent Office), or has been finally decided upon by the Icelandic Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

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NETHERLANDS

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

NORWAY

The applicant hereby requests that, until the application has been laid open to public inspection (by the Norwegian Patent Office), or has been finally decided upon by the Norwegian Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Norwegian Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Norwegian Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Norwegian Patent office or any person approved by the applicant in the individual case.

SINGAPORE

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27.1

Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 23, line 23.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit: 16 April 1999 (16.04.99)	Accession Number: DSM ACC2397

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

MC2 20 22/12/98

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

27.2

Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 20, line 27.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit: 16 April 1999 (16.04.99)	Accession Number: DSM 12777

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

pDHFR

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

27.3

Applicant's or agent's file reference number 1792.002PC02	International application No. TBA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 20, line 14.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depository institution

DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depository institution *(including postal code and country)*

Mascheroder Web 1b
D-38124 Braunschweig

Date of deposit: 16 April 1999 (16.04.99)	Accession Number: DSM 12776

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

pVex 1

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 August 2000 (08.08.00)	
International application No. PCT/US99/26238	**Applicant's or agent's file reference** 1792.002PC02
International filing date (day/month/year) 08 November 1999 (08.11.99)	**Priority date** (day/month/year) 06 November 1998 (06.11.98)
Applicant CARCAGNO, Carlos, Miguel et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 May 2000 (30.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Zakaria EL KHODARY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38